# **Supporting Information**

## Cannata et al. 10.1073/pnas.0710433105

#### SI Text

In Vitro Cleavage Assay. We used the pcDNA-T/S plasmid (5900 bp) or a 29-bp-long DNA intramolecular duplex containing the oligopyrimidine—oligopurine target sequence for triplex formation. The DNA substrate was incubated with OP-TFO/LNA and 20  $\mu$ M CuSO<sub>4</sub> in 50 mM Hepes (pH 7.5), 10 mM MgCl<sub>2</sub>, 50 mM NaCl; the cleavage reaction was started by addition of 3 mM MPA (3-mercaptopropionic acid) and performed at 37°C. The reaction was stopped by adding 250  $\mu$ M 2,9-dimethyl-1,10-phenanthroline (neocuproine).

The supercoiled pcDNA-T/S plasmid was linearized with the restriction enzyme SacII after the cleavage reaction. The cleavage products were analyzed on 0.8% agarose gels (TAEx1) stained with ethidium bromide. The 29-bp duplex was endlabeled by using  $T_4$  polynucleotide kinase and  $\gamma$ -[ $^{32}$ P]ATP. Cleavage products were analyzed on 15% denaturing polyacrylamide gel (TBEx1).

OP-TFO/LNA transfection into HeLa/luc cells and immortalized fibroblasts was performed by strepolysin O-mediated permeabilization (Fig. S2). These two cell lines were described in refs. 22 and 28, respectively. Streptolysin-O (SLO) (a gift from S.Bhakdi, Johannes Gutenberg-Universitat, Mainz, Germany) was used to reversibly permeabilize thes two cell lines. The protocol used is a modification of a previously described SLO-based method (35). Briefly, cells were washed twice in HBSS without Ca<sup>2+</sup> (GIBCO), and 2  $\times$  106 cells were resuspended in 100  $\mu$ l of HBSS. An optimized amount of SLO and 10  $\mu$ l of OP-TFO/LNA (100×) were added for a 15-min incubation at 37° to allow SLO-induced permeabilization. DMEM (0.9 ml) containing 1.8 mM Ca<sup>2+</sup> were then added to reseal cells.

1. Porteus MH, Carroll D (2005) Nat Biotechnol 23:967–73.

2. Alwin S, et al. (2005) Mol Ther 12:610-7.

Chromatin Immunoprecipitation. Confluent monolayers of HeLa/ luc cells were fixed with formaldehyde (1% vol/vol) for 10 min at 37°C. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M. Cross-linked cells were harvested and washed in PBS. Subsequent procedures were performed on ice, with buffers supplemented with protease inhibitor mix (Roche Diagnostics). Cells were lysed in lysis buffer (5 mM Pipes (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40) and incubated on ice for 10 min (with magnetic stirring). Nuclei were pelleted and lysed by incubation in nuclear lysis buffer [50 mM Tris·HCl (pH 8.1), 10 mM EDTA, 1% SDS]. Sonication was performed with a Bioruptor type sonicator (Diagenode) in closed 1.5-ml tubes. Settings of the sonicator were: first cycle (15 min) at medium intensity: 20 sec On/ 1 min Off; second cycle (15 min) at high intensity: 10 sec ON/ 1 min OFF; third cycle (6 min) at high intensity: 20 sec ON/1 min OFF.

DNA contents in nuclear extracts were standardized after quantification by using a NanoDrop UV-Vis. spectrophotometer. After centrifugation, the supernatant was diluted 10-fold with dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM TrisHCl (pH 8.1), 167 mM NaCl]. Diluted extracts were precleared with protein A/protein G agarose beads (Sigma) and incubated with anti-phosphoH2AX antibodies (Upstate), and immunoprecipitated with protein A/protein G agarose beads (Sigma). After extensive washing, bound DNA fragments were eluted by overnight incubation at 65°C and purified by treatment with proteinase K, phenolchloroform extraction, and ethanol precipitation. Samples were analyzed by quantitative PCR (Mx3005P Real-Time PCR System Stratagene) using specific primers (see sequences in Table S1).

3. Bibikova M, Golic M, Golic KG, Carroll D (2002) Genetics 161:1169-75.

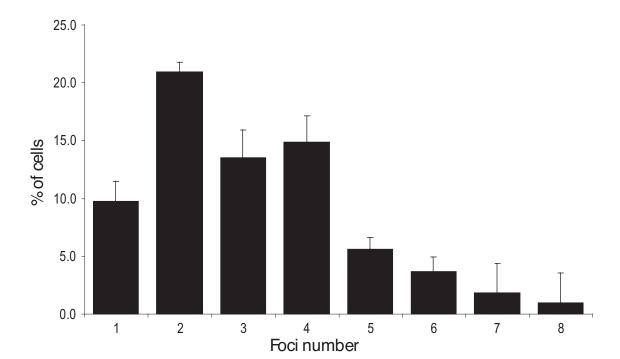


Fig. S1. Detailed quantification of phosphoH2AX foci number 24 h after OP-19-mer TFO/LNA treatment (corresponding to data from Fig. 2*A Right*).

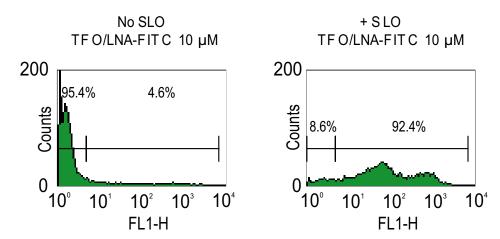


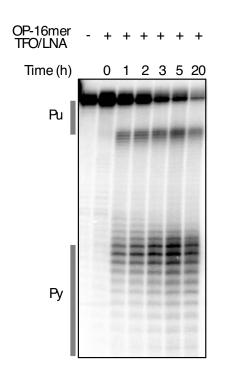
Fig. S2. Evaluation of transfection efficiency of OP-TFO/LNA conjugates. FACS analysis of cells was performed 1 h after SLO permeabilization of HeLa/luc cells in presence of 10  $\mu$ M TFO/LNA-FITC. Dual-parameter flow cytometry using propidium iodide (added just before FACS analysis), for dead-cell staining, and the fluorescein-labeled oligonucleotide, TFO/LNA-FITC, for monitoring transfected cells, was performed. Transfection efficiency of fluorescein-labeled oligonucleotide was  $\approx$ 90%. Fluorescence microscopy allowed the observation of uniform distribution of TFO/LNA-FITC within cells (data not shown).

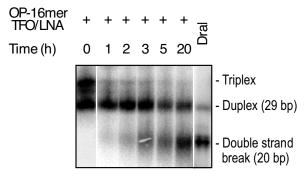
## В

#### Immortalized Fibroblast

Untreated TATGAGAGACAAGICTITAT AAAAAAAGGGGGGGAGAGAGAAAAI B1						98.8%							
		TATGAGAGACAAGTCTTAT	AAA	A G A	АА	A G	GGG	GGG	A G	ΑА	GAAGATGAAT	1	1.2%
	t D	TATGAGAGACAAGTCTTAT	ААА	A G A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAAT	60	87.0%
		TATGAGAGACAAGTCTTAT	АА-	A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAAT	1	1.4%
		TATGAGAGACAAGTCTTT	AAA	A G A	AA	A G	GGG	GGG	A G	A G	G A A G A T G A A T 1	1	1.4%
	<u> </u>	TATGAGAGACAAGTCTTAT	A	A G A	AA	A G	GGG	GGG	A G	A G	GAAGATGAAT	1	1.4%
	Ĕ	TATGAGAGACAAGT TTAT	AAA	A G A	АА	A G	GGG	GGG	A G	A G	GAAGATGAAT	1	1.4%
	÷	TATGAGAGACAAGTCTTAT	AGA	A G A	АА	A G	GGG	G G G	A G	A G	G A A G A T G A A T 1	1	1.4%
	be	TATGAGAGACAAGTCTTTGT	AAA	A G A	АА	A G	GGG	GGG	A G	A G	GAAGATGAATI	1	1.4%
	Experiment	T A T G A G A G A C A A G T C T T A T	AAA	AAA	АА	ΑG	GGG	GGG	A G	A G	G A A G A T G A A T 1	1	1.4%
		TATGAGAGACAAGTCTTAT	AAA	A G A	AA	G G	GGG	GGG	A G	A G	GAAGATGAATI	1	1.4%
		TATGAGAGACAAGTCTTTT	AAA	AGA	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.4%
		TATGAGAGACAAGTCTTAT	AAA	A G A	АА	ΑG	GGG	G G	A G	A G	G A A G A T G A A T 1	84	88.4%
≥		TATGAGAGACAAGTCTTAT	,	A A A	АА	A G	GGG	GGG	A G	A G	G A A G A T G A A T 1	1	1.1%
50µM		TATGAGAGACAAGTCTTTA -	- A A	A G A	АА	A G	GGG	G G	A G	A G	G A A G A T G A A T 1	1	1.1%
	ш	T A T G A G A G A C A A G T T T A T	AAA	A G A	АА	A G	GGG	GGG	A G	A G	GAAGATGAAT	1	1.1%
TFO/LNA	Experiment	TATGAGAGACAAGTCTTT	AAA	A G A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.1%
Z/	l e	TATGAGAGACAAGTCTT-AT	AAA	A G A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.1%
9	I ⋅⊑	TATGAGAGACAAGTCTTAT	A	A G A	АА	A G	GGG	GGG	A G	A G	GAAGATGAATI	1	1.1%
OP-19mer T	)e	TATGAGAAACAAGTCTTAT	AAA	AGA	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.1%
	×	TATGAGAGACAAGTCTTAT	AAA	A G A	АА	ΑΑ	GGG	GGG	A G	A G	GAAGATGAATI	1	1.1%
	Ш	TATGAGAGACAAGTCTTAT	AAA	A G A	АА	ΑG	GGG	GGG	G G	A G	G A A G A T G A A T 1	1	1.1%
		TATGAGAGACAGGTCTTAT	AAA	A G A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.1%
		TATGAGAGACAAGTCTTAT	AGA	AGA	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.1%
		TATGAGAGACAAGTCTTAT	AAA	A G A	АА	ΑG	GGG	G G G	A G	A G	G A A G A T G A A T 1	71	89.9%
	Щ.	TATGAGAGACAAGTCTTAT	A	A G A	АА	A G	GGG	GGG	A G	A G	GAAGATGAATI	1	1.3%
	Ξ.	TATGAGAGACAAGTCT AT	AAA	A G A	АА	ΑG	GGG	3 G G	A G	A G	GAAGATGAATI	1	1.3%
	иe	TATGAGAGACAAGTCTTAT	ААА	G G A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.3%
	=	TATGAGAGACAAGTCTTTT	AAA	A G A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.3%
	Experiment	TATGAGAGACAAATCTTAT	AAA	A G A	АА	ΑG	GGG	GGG	A G	A G	GAAGATGAATI	1	1.3%
		TATGAGAGACAAGTCTTAT	AAA	A G A	АА	ΑG	GGG	GGG	АА	A G	GAAGATGAATI	1	1.3%
	Ι "	TATGAGAGACAAGTCTTAT	AGA	A G A	AA	A G	GGG	3 G G	A G	A G	G A A G A T G A A T 1	1	1.3%
	1	T A T G A G A G A C A A G T C T T A T	AAA	AGA	AA	G G	GGG	GG	A G	A G	GAAGATGAAT	1	1.3%

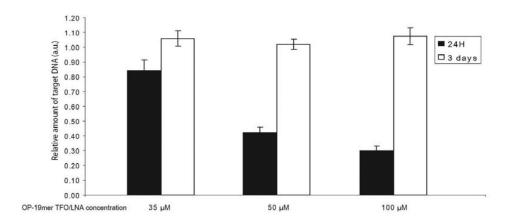
**Fig. S3.** Mutations patterns 72 h after treatment with OP-19-mer TFO/LNA (50 μM during the SLO-permeabilization procedure). Three independent experiments were performed in HeLa cells (*A*–*C*) and in human immortalized fibroblasts (*D* to *F*), respectively. These data correspond to the histogram presented in Fig. 3C. The OP-19-mer TFO/LNA target sequence is indicated in bold.





**Fig. S4.** Cleavage activity of OP-16-mer TFO/LNA conjugate on a synthetic DNA target. An intramolecular duplex (29 bp) was used as a target after 5' radiolabeling. Cleavage products were analyzed by denaturing (*Left*) or nondenaturing (*Right*) PAGE. A schematic representation of the corresponding cleavage pattern is depicted in Fig. 1 in the main text.





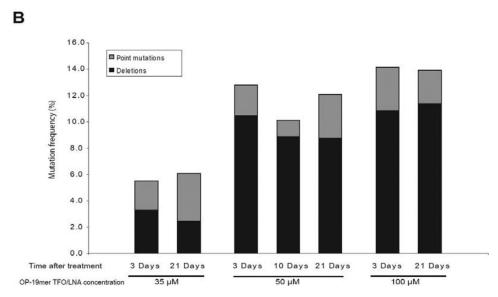


Fig. S5. Dose-dependent activity of OP-19-mer TFO/LNA. (A) Dose-dependent induction of DSBs at the target site. PCR quantification of the target DNA was performed by using primers flanking the OP-19-mer TFO/LNA-binding site, at the indicated times after treatment of HeLa/mutGFP-19TF cells with the indicated concentration of OP-19-mer TFO/LNA conjugate. The values were normalized by quantification of a control genomic sequence where no triplex site is available. (B) Dose-dependent induction and long-term stability of mutations at the target site The target region was amplified at 3, 10, and 21 days after treatment of HeLa/mutGFP-19TF cells with the indicated concentration of OP-19-mer TFO/LNA conjugate and individual PCR products sequenced after cloning. (C) Sequencing results. The indicated concentrations correspond to the ones used during the SLO permeabilization procedure (see Materials and Methods).

•				
Untre	ated	-10 -9 -8 -7 -6 -5 -4 -3 -2 -1	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  A A A A G A A A A G G G G G G A G A G	100.0%
		TCCAAGCTTT	<b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A <b>6</b> 5 <b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A <b>8</b> 6	94.5%
OP-19mer TFO/LNA 35 µM	/2	TCCAAGCTTT	AAGAAAAGGGGGAGAGGCAAATTTGTA 1	1.1%
	Days	TCCAAGCT	AAAAGAAAAGGGGGGAGAG	1.1%
	3	T C C A A T T T	AAAAGAAAAGGGGGAGAGGCAAATTTGTA 1	1.1%
		T C C A A G G T T T T C C A A G C T T A	AAAAGAAAAGGGGGGAGAGGCAAATTTGTA 1 AAAAGAAAAGGGGGGAGAGGCAAATTTGTA 1	1.1%
		TCCAAGCTTA	AAAAGAAAGGGGGGAGAGGCAAATTTGTA 77	93.9%
9m 35	Days	T C C A A T	AAAAGAAAGGGGGGAGAG	1.2%
-1.	$\tilde{\Box}$	T C C T T T	<b>AAAAGAAAGGGGGGAGAG</b> GCAAATTTGTA 1	1.2%
0	21	T C C A A C C T T T	A A A A G A A A A G G G G G G A G A G G C A A A T T T G T A 1	1.2%
		T C A A A G C T T T T C C A A G G T T T	<b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A 1 <b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A 1	1.2%
		TCCAAGCTTT	AAAAGAAAGGGGGGAGAGGCAAATTTGTA 75	87.2%
		TCCAAGCTT-	AAAAGAAAGGCGG- AGAGGCAAATTTGTA 1	1.2%
		T C C A A G C T	- AAGAAAAGGGGGAGAG	1.2%
		T C C A T T T	AAAAGAAAAGGGGGGGAGAGGCAAATTTGTA 1	1.2%
	3 Days	T C A G C T T T T C C A A G C T T -	AAAAGAAAAGGGGGGAGAGGCAAATTTGTA 1 AAGAAAAGGGGGAGAGGCAAATTTGTA 1	1.2% 1.2%
		TCCAAGCTTT	- AAAGAAAAGGGGGGAGAGGCAAATTTGTA 1	1.2%
		G C T T T	AAAAGAAAAGGGGGGAGAG	1.2%
		T C C A	- AAAGAAAAGGGGGAGAGGCAAATTTGTA 1	1.2%
		T C C	A G G G G G G A G A G G C A A A T T T G T A 1 G G A A G A A A A G G G G G G A G A G G C A A A T T T G T A 1	1.2%
		TCCAAGCITI	A A A G A A A A G G G G G G A G A G G C A A A T T T G T A 1	1.2%
∢		TCCAAGCTTT	AAAAGAAAAGGGGGGAAAGGCAAATTTGTA 71	89.9%
OP-19mer TFO/LNA 50 µM		T C C A A G	<b>AGAAAAGGGGGAGAG</b> GCAAATTTGTA 1	1.3%
ner TFO/I 50 µM	/8	T C C A A G C T T -	AAAAGAAAAGGGGGGAGAGGCAAATTTGTA 1	1.3%
투 의	10 Days	T C C A A T T T C C A A G C T T T	<b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A 1 <b>A A G A A A A G G G G G A G A G</b> G C A A A T T T G T A 1	1.3%
me 5	] 0	T C C A A G C I I I	<b>A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A <b>1</b> <b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A <b>1</b>	1.3%
-19	1	TCCAAGCTT-	GAAAAGGGGGGAGAG	1.3%
e l		T C C A A G	<mark>-GAAAAGGGGGGAGAG</mark> GCAAATTTGTA 1	1.3%
		TCCAAGCTTT	AAAGGAAAAGGGGGAGAGGCAAATTTGTA 1	1.3%
		T C C A A G C T T T T C C A A T	AAAAGAAAAGGGGGGAGAGGCAAATTTGTA 80 AAAAGAAAAGGGGGGGAGAG	87.9% 1.1%
		T C C A A T T T	AAAAGAAAGGGGGGAGAGGCAAATTTGTA 1	1.1%
		TCCAAGCT	GAAAGGGGGGAGAGGCAAATTTGTA 1	1.1%
	21 Days	T C C	AAGAAAGGGGGGAGAG	1.1%
	Ö	G C T T T	<b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A 1	1.1%
	7	T C C A A	G A A A A G G G G G G A G A G G C A A A T T T G T A 1 A A A A G A A A A G G G G G G A G A G G C A A A T T T G T A 1	1.1%
		T C C A A G C T T -	AAGAAAAGGGGGGAGAGGCAAATTTGTA	1.1%
		TCCAGGCTTT	AAAAGAAAAGGGGGGAGAG	1.1%
		TCCAAGCCTT	AAAAGAAAGGGGGGAGAG	1.1%
	3 Days	TCCAAGCTTT	TAAAGAAAAGGGGGGAGAGGCAAATTTGTA 1 AAAAGAAAGGGGGGGAGAGGCAAATTTGTA 79	1.1%
		T C C A A G C T T T	AAAAGAAAAGGGGGGAGAGGCAAATTTGTA 79	85.9% 1.1%
		T C C A	- AAAGAAAGGGGGGAGAG	1.1%
		A G C T T T	AAAAGAAAAGGGGGGAGAG	1.1%
		TCCAAGCTTT	A A A A G G G G G G A G A G G C A A A T T T G T A 1	1.1%
		T C C A A 1 1	<b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A 1	1.1%
		T C C A C T T T	A A A G A A A A G G G G G G A G A G G C A A A T T T G T A 1	1.1%
		T C C A A G C	AAAAGAAAAGGGGGAGAG	1.1%
OP-19merTFO/LNA 100 µM		TCCAAGCTT-	AAGAAAAGGGGGAGAG	1.1%
			AAAAGAAAAGGGGGGAGAGGCAAATTTGTA 1 AAAAGAAAGGGGGGGAGAG	1.1%
0		T C C A A G G T T T T C C A A G C T T T	<b>A A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A <b>1 T A A A G A A A A G G G G G G A G A</b> G C C A A A T T T G T A <b>1</b>	1.1%
<u></u>		TCCAGGCTTT	A A A G A A A G G G G G G A G A G C A A A T T T G T A 1	1.1%
19mer TF 100 µM		TCCAAGCTTT	AAAAGAAAAGGGGGGAGAG	86.1%
19	21 Days	T T T	AAAAGAAAAGGGGGAGAGGCAAATTTGTA 1	1.3%
<u>ظ</u>		T C C A A	<b>A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A	1.3%
J		T T	- <b>A A A G A A A A G G G G G G A G A G</b> G C A A A T T T G T A <b>1</b> <b>A A A A G A A A A G G G G G G A G A</b> G C C A A A T T T G T A <b>1</b>	1.3%
		T C C A A G C T	GAAAAGGGGGGAGAGGCAAATTTGTA 1	1.3%
		A G C T T T	AAAAGAAAGGGGGGAGAG	1.3%
		TCCAAGCTTT	A A A A G G G G G G A G A G G C A A A T T T G T A 1	1.3%
		T C C A A G T C C A A G C T T -	<b>A A A A G G G G G G A G A G</b> G C A A A T T T G T A	1.3%
		TCCAAGCTTA	A A A A G A A A A G G G G G G A G A G C A A A T T T G T A 1	1.3%
			AAAAGAAAAGGGGGGAGAG	1.3%
			Fig. S5. Continued	

Fig. S5. Continued.

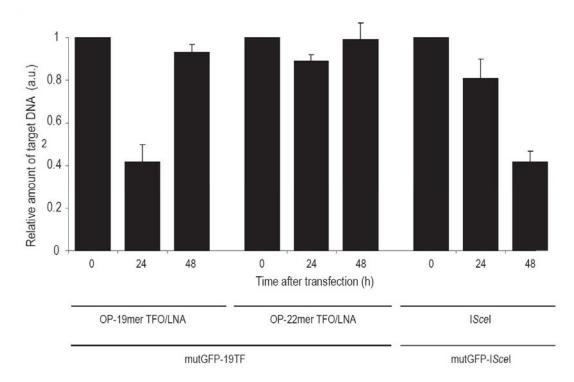


Fig. S6. Target sites that have been inserted at the eGFP locus are efficiently cleaved in HeLa/mutGFP-19TF or HeLa/mutGFP-IScel cells after OP-19-mer TFO/LNA or IScel expression plasmid transfection, respectively. Cells were transfected in conditions of Fig. 4 and the DNA target region was analyzed by quantitative PCR. The values were normalized by quantification of a control genomic sequence. Interestingly, targeted cleavage was still detected at 48 h after transfection for IScel, but not OP-19-mer TFO/LNA-transfected cells. This likely reflects persistent ISce1 expression and activity from the transfected plasmid as opposed to transient cleavage activity from the OP-TFO/LNA conjugates. Persistent cleavage activity in cells expressing protein-based nucleases may contribute to toxicity (1–3).

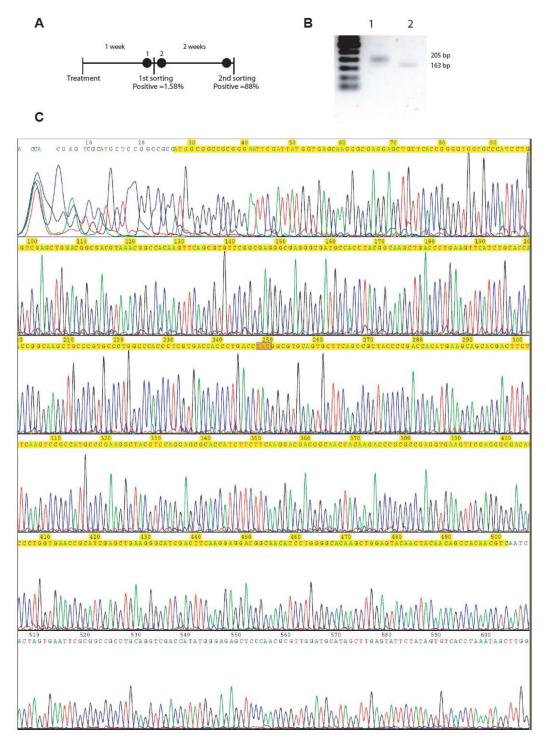


Fig. S7. Analysis of the chromosomal reporter gene sequence after OP-19-mer TFO/LNA and donor transfection. (A) Time line of events. The two FACS sorting were indicated by vertical bars. (B) One week after transfection, PCR amplification of the target sequence was performed on cells (black circle 1) and on green-fluorescent cells isolated by FACS (black circle 2), and PCR products were analyzed by agarose-gel electrophoresis (lanes 1 and 2, respectively). The 205-bp fragment corresponds to amplification of the mutGFP-19TF cDNA sequence, whereas the 163-bp fragment corresponds to the correction of mutGFP-19TF into GFP cDNA sequence. (C) Three weeks after transfection, green-fluorescent cells were isolated by FACS. Just before sorting (black circle 3) sequencing of the target sequence were performed after PCR amplification and cloning. Twenty two of 24 clones contained the wild-type GFP sequence; the chromatogram of one of these 22 clones is represented. The triplex site inserted at codon 65 to construct mutGFP-19TF cDNA is no longer present, and the mutGFP-19TF sequence has been corrected into eGFP cDNA as expected (position of codon 65 is indicated by a box). The two other clones contained the sequence present in the mutGFP-19TF DNA and resulted from imperfect sorting because 2 of 24 = 8% is consistent with the 88% of green cells obtained in the second sorting.

#### Table S1. Primer sequences

Primer sequence (5'-3'	Amplified region	Amplicon size, bp
CTGCCTGCAGGAGAAAAGAA	Chr 7 around the OP-19-mer TFO/LNA target sequence (for qPCR)	191
CCCTCCCATTTCAAAGTCAG		
TGATAATAGAGCCAATCCCTCA	Chr 7 around the OP-19-mer TFO/LNA	405
TTTGTCCCATTCTGTTTTATTTCA	target sequence (for junction analysis)	
TCCCACTTCTGAAAAGTCTGTACT	Chr 7, 3 kbp from the OP-19-mer TFO/LNA	172
AAAGAGAAACTGAGAGGAAAAATCA	target sequence (for ChIP)	
CCACTCCTGATTTCTGGAAAAG	GAPDH (for ChIP)	171
GAAATTAACTGGACAGGGCAAG		
ATGGTGAGCAAGGGCGAG	Integrated mut eGFP (for sequence analysis)	513
GACGTTGTGGCTGTTGTAGTTG		
CCTCGTGACCACCCTGAC	Integrated mut eGFP (for qPCR)	107
GGTAGCGGCTGAAGCACT		
ACATGAAGCAGCACGACT	Reference region (for qPCR)	93
TCTTGTAGTTGCCGTCGT		